QUALITY CONTROL

An integral part of the Penn Vector Core is its robust quality control program which is carried out by a separate quality control group. Quality control assays have been developed and optimized for each vector and criteria are in place which must be met before the release of any vector from our facility. Standard operating procedures (SOPs) are followed and batch record forms utilized for each assay conducted by the Core.

The outcome of gene therapy research can be dramatically influenced by the quality of viral vector used. The Quality Control (QC) Core provides numerous services to assess the quantity, quality and purity of adenovirus, adeno-associated virus (AAV), and lentivirus vectors produced by the Penn Vector Core, investigators at the University of Pennsylvania and external collaborators. The mission of the QC Core is to provide accurate information on the quality of viral vectors provided by the Penn Vector Core and to continually strive to improve the accuracy and scope of services offered by developing and optimizing novel assays useful for assessing the efficacy of gene therapy vectors.

Quality Control Assays

AAV Vectors

**Plasmid Characterization:** Three types of plasmids are used in AAV vector production: AAV cis plasmids, containing the vector genome, AAV trans plasmids containing rep and cap genes, and adenovirus helper plasmids encoding essential adenovirus functions. A critical assessment of quality is confirmation of plasmid identity, and, therefore, a plasmid identity assay as well as additional quality control testing is conducted by the QC Core. Full plasmid characterization includes determination of concentration, determination of A260/280 ratio, detection of endotoxin, and plasmid identity. In the plasmid identity assay, the presence of each functional DNA element is determined by sequencing and the correct restriction pattern is verified with the plasmid map and the seed DNA preparation. For AAV cis-plasmids, these elements include the 5’ and 3’ ITR, transgene, promoter, polyadenylation sequence and any other regulatory element. For AAV trans plasmids, the presence of the correct cap gene is verified by full length sequencing.

**Genome Copy (GC) Number Titration**

Real time PCR using TaqMan (Applied Biosystems, Foster City, CA) reagents and machines are employed to determine the genome copy (GC) number of AAV vector lots as a measure of AAV particles with full genome content. The accuracy and reliability of the quantitative PCR GC titration is paramount for AAV QC and affects the outcome of other downstream assays. For this reason, the QC Core has put extensive effort into ensuring that the genome titrations are conducted with precision and consistency (Figure 1). A number of standards, validation samples (both viral and plasmid) and controls (for background and DNA contamination) have been introduced into the assay. The QC Core demonstrates an impressive turn-around time for genome titration and maintains stocks of primers and probes designed for vector titration in response to rapid advancement in vector designs.
Figure 1. Reproducibility of real time PCR titration for a validation sample Z504 carrying an AAVLacZ genome with a bovine growth hormone poly A which is the target for PCR amplification. The Y-axis represents the genome titers of each RCR run, while the X-axis indicates numbers of titration performed. Each preparation is diluted in a constant volume so that concentration is directly proportional to yield.

Infectious titer determination (Taqman TCID50)

In order to improve sensitivity, reproducibility and turn-around time, an infectivity assay for AAV vectors has been developed and used as an alternative to standard techniques such as the infectious center assay (ICA). The Taqman TCID50 assay is based upon limiting dilution of the vector and a 50% endpoint determination of viral DNA replication using real-time PCR for sensitive, quantitative calling of positive wells. AAV vectors are serially diluted and a cell line expressing AAV rep and cap is co-infected with these dilutions plus wildtype Ad5 in a 96-well plate format (8 replicate wells per dilution). The presence of AAV rep and adenovirus helper genes allows for the replication of AAV DNA. After a suitable incubation period, DNA is extracted and a 50% endpoint determination is performed by a basic computer program based upon Karbers formula. With a validation sample included in every run, the assay has proven to be accurate and reproducible. A GC: infectivity (GC:I) ratio is calculated based upon the results of both the GC copy number titration and TCID50 assay results. The GC:I ratio is used as a measure of infectivity of the preparation with low GC:I ratios indicating more infectious vector lots. The GC:I ratios between different lots of the same serotype are useful in assessing the relative potency of a particular preparation. However, the infectivity of a vector based on GC:I ratio does not predict the in vivo performance, particularly when comparing different AAV serotypes.

Purity (SDS-PAGE)

The method used to purify an AAV vector can dramatically influence the purity of the preparation in terms of the amount of host cell protein contamination. The purity of a vector preparation can be analyzed by SDS-polyacrylamide gel electrophoresis. Bands corresponding to
the viral structural proteins, VP1, VP2 and VP3 may be visualized by SYPRO Ruby staining and their size and relative intensity assessed with respect to contaminating proteins.

Figure 2. SDS-PAGE and purity assessment of AAV vector preparation. AAV proteins were separated by SDS-PAGE and then detected by staining with SYPRO Ruby (Invitrogen). Fluorescence intensity of the protein bands were quantified by Syngene gel imaging system using GeneTools Software. a) BML, BenchMark molecular weight ladder, Lanes 1-3: 3 lots of AAV vector. The position of the AAV virion proteins VP1, VP2 and VP3 are indicated. Minor contaminating band in vector lot 2 below the virion proteins is visible. b)Table shows results of capsid protein quantification indicating that AAV virion proteins comprise >90% of the detected proteins.

Endotoxin Assay (Limulus Amebocyte Lysate)

The purpose of this assay is to detect and quantitatively determine the gram-negative bacterial endotoxin level in plasmid or vector preparations. Contaminating endotoxin in plasmid preparations can affect transfection efficiencies as described above. Endotoxin contamination in vector preparations can alter the immunogenic properties of the final product. The endotoxin assay is carried out using the Limulus Amebocyte Lysate (LAL) gel-clot method (QCL-1000, Bio Whittaker). Release criteria for vector lots to be used in large animal studies are < 5
endotoxin units per ml. Preparations exceeding the endotoxin release criteria are discarded and the vector is re-prepared.

**Adenoviral Vectors**

**Physical Particle Number Determination**

The physical particle titer of adenoviral vectors is determined using absorbance readings at 260nm and an extinction coefficient (E260) of 1.1x1012 vector particles per A260 unit as determined by Maizel et al. (Maizel et al., 1968) for AdHu2. An A280 reading is also taken and A260/A280 ratios of ~1.3 are consistently obtained for our vector preparations. Ratios higher than this are an indication of cellular DNA contamination in the preparation; lower ratios indicate protein contamination.

**Infectious titer determination (Taqman TCID50)**

In order to improve sensitivity, reproducibility and turn-around time, an infectivity assay for adenoviral vectors has been developed and used as an alternative to standard techniques such as the plaque assay. The Taqman TCID50 assay is based upon limiting dilution of the vector and a 50% endpoint determination of viral DNA replication using real-time PCR for sensitive, quantitative calling of positive wells. Briefly, adenoviral vectors are serially diluted (10-fold dilutions) and used to infect 293 cells in a 96-well plate format (8 replicate wells per dilution). After a three day incubation period, replicated DNA is extracted and quantified using real-time PCR primer-probe sets specific for the E2a gene. A 50% endpoint determination is performed by a basic computer program which is based upon Karbers formula. Validation of the assay involving several runs each of different vectors shows excellent reproducibility of titer (IU/ml) and particle to infectivity (P:I) ratios (Table 1). The P:I ratio is used as a measure of the infectivity of the preparation with low P:I ratios indicating more infectious vector preps. The assay consistently returns P:I ratios which are much lower than those achieved with the plaque assay or with TCID50 assays based on observation of cytopathic effects (CPE). Taqman TCID50 derived P:I ratios are most useful as a measure of lot to lot consistency within a vector subtype.

<table>
<thead>
<tr>
<th>Assay</th>
<th>H5.040.CMV/transgene A</th>
<th>H5.040.CMV/transgene B</th>
<th>H5.040.CMV/transgene C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TCID50 IU/ml</td>
<td>SE (%)</td>
<td>P/I ratio</td>
</tr>
<tr>
<td>1</td>
<td>6.32x10^11</td>
<td>31.6</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>1.12x10^12</td>
<td>29.7</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>6.32x10^11</td>
<td>31.6</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>4.74x10^11</td>
<td>35.8</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>3.56x10^11</td>
<td>29.7</td>
<td>17</td>
</tr>
<tr>
<td>mean</td>
<td>6.43x10^11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>SD</td>
<td>2.91x10^11</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 1. Taqman TCID50 validation runs
Replication-competent adenovirus (RCA) assay

Replication-competent adenoviruses can emerge during the creation and propagation of E1-deleted replication-defective adenoviral vectors as a result of recombination between AdHu5 vector sequences and overlapping human Ad5 E1 gene/flanking sequences present in 293 cells. Depending on the level of RCA in vector preparations, significant impact on vector performance, host immune responses and toxicological profiles can be observed in *in vivo* experiments. Therefore, identification of vector preparations with a high level of RCA contamination is critical for gene transfer and vaccine applications using adenoviral vectors.

We currently run two types of RCA assay:

a) Routine CPE detection assay. This assay is sufficient for most research purposes and ensures that high levels of RCA are not present in the vector preparation. The level of sensitivity of the routine RCA assay for AdHu5 is 10 RCA in 109 particles of test article. The assay spans a 2 week period and the readout is the observation of characteristic CPE. Wild-type H5 virus is spiked into test articles at various concentrations to determine the sensitivity of each assay and, in cases where RCA is present, to give a semi-quantitative assessment of contamination levels.

b) Real-time PCR detection assay. This assay is in place for adenoviral vectors where a high level of sensitivity for detection of RCA is required. The assay is similar to the routine CPE detection assay described above except, in this case, RCA is amplified by passaging and then detected by real-time PCR using E1 specific primers and probes. This substantially improves the sensitivity of the assay to 1 IU RCA in 1011 particles.

Genome structure analysis

A detailed restriction endonuclease digest analysis of vector genomes is performed to confirm the identity and integrity of the vectors. Since our vectors are all created from molecular clones followed by rescue and expansion in 293 cells, the molecular clone and the vector backbone plasmid used for generation are analyzed in side-by-side restriction enzyme digestions with extracted vector DNA. The resulting fragments are separated by electrophoresis in agarose gels, ethidium bromide stained and the signature banding patterns are compared. At least two sets of restriction enzymes are usually selected for analysis. One set focuses on detecting the presence and integrity of minigene cassettes while the other set emphasizes vector backbones. An example of the use of this type of assay in detecting a subpopulation of rearranged AdHu5LacZ preparation is shown in Figure 2. In this case, the rearrangement appeared as a minority population after passaging the master seed stock of the AdHu5LacZ vector twice. The normal expansion scheme (one passage from the master seed) used for expanding adenoviral vectors, did not result in rearrangement.
Figure 3. Restriction endonuclease analysis of AdHu5LacZ vector preparations. DNA extracted from 5 separate AdHu5LacZ preparations (A-E) was restricted with two sets of enzymes and the fragments separated on an agarose gel. Vectors purified after one round of passage from the master seed stock (A and B) show the expected sized fragments predicted by the vector map whereas those purified after two rounds of passage (C, D and E) contain unpredicted fragments (arrows) and indicate a sub-population with a rearranged backbone.

Endotoxin Assay (Limulus Amebocyte Lysate)

The purpose of this assay is to detect and quantitatively determine the gram-negative bacterial endotoxin level in plasmid or vector preparations. Contaminating endotoxin in plasmid preparations can affect transfection efficiencies as described above. Endotoxin contamination in vector preparations can alter the immunogenic properties of the final product. The endotoxin assay is carried out using the Limulus Amebocyte Lysate (LAL) gel-clot method (QCL-1000, Bio Whittaker). Release criteria for vector lots to be used in large animal studies are < 5 endotoxin units per ml. Preparations exceeding the endotoxin release criteria are discarded and the vector is re-prepared.

Lentiviral / Retroviral Vectors

Infectious DNA titer assay

In support of our expanding lentiviral program, a generic approach for assessing infectious titer has been adapted for lentiviral vectors based on the work of Sastry et al. (Sastry et al., 2002). Real-time PCR and a primer probe set specific for the 5’ untranslated region of the vector is used to detect proviral genomic DNA as a measure of infectious viral particles entering the cell. 96 well plates containing 293 cells are infected with 10-fold serial dilutions of the vector and after 3
days of incubation, genomic DNA is extracted and assayed for proviral DNA content using real-time PCR. Those dilutions giving vector genome copy numbers over 100 are used to calculate the titer. The assay readout is linear over a 5 log dilution and has given reproducible titers for validation vectors. This method of assessing titer is ~10 fold more sensitive than GFP transduction-based techniques when using a GFP reporter vector. Furthermore this approach is independent of the transgene. This method can be adapted to the estimate of titer for retroviral vectors.

**Replication-competent lentivirus (RCL)**

Lentiviral vector stocks are tested for the presence of replication-competent lentivirus (RCL) by monitoring p24 antigen expression in the culture medium of transduced 293T cells for 30 days. Serial passaging of the transduced p24 cells over this period allows for the amplification of RCL. Since the vectors are replication-defective, no amplification of p24 signal would normally be expected, however, an increasing P24 signal over time would be indicative of RCL contamination of the vector lot. This assay has been performed on multiple lots of vector without a positive result. A standard mobilization assay is used to assess the presence of RCR in retroviral preparations.

**Endotoxin Assay (Limulus Amebocyte Lysate)**

The purpose of this assay is to detect and quantitatively determine the gram-negative bacterial endotoxin level in plasmid or vector preparations. Contaminating endotoxin in plasmid preparations can affect transfection efficiencies as described above. Endotoxin contamination in vector preparations can alter the immunogenic properties of the final product. The endotoxin assay is carried out using the Limulus Amebocyte Lysate (LAL) gel-clot method (QCL-1000, Bio Whittaker). Release criteria for vector lots to be used in large animal studies are < 5 endotoxin units per ml. Preparations exceeding the endotoxin release criteria are discarded and the vector is re-prepared.